Isoaspartyl Formation in Creatine Kinase B Is Associated with Loss of Enzymatic Activity; Implications for the Linkage of Isoaspartate Accumulation and Neurological Dysfunction in the PIMT Knockout Mouse

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Abstract

Isoaspartate (isoAsp) formation is a common type of spontaneous protein damage that is normally kept in check by the repair enzyme protein-L-isoaspartyl methyltransferase (PIMT). PIMT-KO (knockout) mice exhibit a pronounced neuropathology highlighted by death from an epileptic seizure at 30 to 60 days after birth. The mechanisms by which isoaspartyl damage disrupts normal brain function are incompletely understood. Proteomic analysis of the PIMT-KO mouse brain has shown that a number of key neuronal proteins accumulate high levels of isoAsp, but the extent to which their cellular functions is altered has yet to be determined. One of the major neuronal targets of PIMT is creatine kinase B (CKB), a well-characterized enzyme whose activity is relatively easy to assay. We show here that (1) the specific activity of CKB is significantly reduced in the brains of PIMT-deficient mice, (2) that *in vitro* aging of recombinant CKB results in significant accumulation of isoAsp sites with concomitant loss of enzymatic activity, and (3) that incubation of *in vitro* aged CKB with PIMT and its methyl donor S-adenosyl-L-methionine substantially repairs the aged CKB with regard to both its isoAsp content and its enzymatic activity. These results, combined with similarity in phenotypes of PIMT-KO and CKB-KO mice, suggests that loss of normal CKB structure and function contributes to the mechanisms by which isoAsp accumulation leads to CNS dysfunction in the PIMT-KO mouse.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Introduction

Creatine kinases (EC 2.7.3.2) are a family of enzymes that catalyze reversible transfer of the phosphate group between ATP and creatine, playing a crucial role in intracellular energy metabolism of cells with high and fluctuating energy requirements [1–3]. There are four major isozymes of creatine kinase that are products of separate genes; two cytosolic forms, predominant in brain (CKB) or muscle (CKM), and two mitochondrial forms (the ubiquitous and sarcomeric CKs). CKB is essential for optimal performance of central nervous system (CNS) function, and defects in creatine metabolism have been associated with a wide range of neuropathology in humans [4]. Knockout of the CKB gene in mice results in a phenotype with impaired learning, diminished habituation, abnormal microstructure in the hippocampus, and increased susceptibility to epileptic seizures [5]. Deficiency of CKB activity in human neurological disease has been attributed mainly to oxidative damage [6–8].

In 2006 we reported a proteomics study suggesting that CKB is highly susceptible to spontaneous formation of abnormal iso-aspartyl (isoAsp) residues *in vivo* [9]. Formation of isoAsp is considered to be a deleterious modification that can significantly

impact protein activity and elicit autoimmunity [10–14]. IsoAsp arises from deamidation of asparagine or dehydration of aspartic acid, leading to formation of a metastable succinimide (cyclic imide) intermediate that hydrolyzes to a mixture of aspartyl and isoaspartyl linkages (Fig. 1). IsoAsp formation occurs most readily at sequences in which the side chain of the C-flanking amino acid is relatively small and hydrophilic. Asx-Gly, Asx-Ser and Asx-His sequences, particularly when located in highly flexible protein regions, constitute "hot spots" for isoAsp formation. Cellular mechanisms for dealing with isoaspartyl protein damage include urinary excretion of the damaged proteins [15], degradation by isoAsp-selective proteases [16,17], and enzymatic repair. Regarding the latter mechanism, isoAsp residues in peptides and proteins are specifically recognized and repaired by the action of protein L-isoaspartyl O-methyltransferase (PIMT, EC 2.1.1.77) [18–23]. PIMT utilizes the cofactor S-adenosyl-L-methionine (AdoMet) to transfer a methyl group onto the α-carboxyl group of isoAsp sites leading to succinimidyl intermediate that subsequently hydrolyze to L-Asp and L-isoAsp (Fig. 1). Continuing cycles of PIMT action efficiently repair L-isoAsp sites *in vitro* [23–25], while reduction of PIMT activity in cultured cells or knockout (KO) mice dramat-

ically increases the level of isoAsp-containing proteins [26–29]. PIMT is widely distributed in mammalian tissues, but is particularly rich in the CNS [30–33]. The critical need for PIMT in brain is evident from the overt neurological phenotype of PIMT-deficient mice; increased brain size, abnormal neuro-anatomical and electrophysiological properties of hippocampal cells along with reduced cognitive function [34], atypical open-field behavior [35], and fatal epileptic seizures beginning at 4 weeks of age [27,28]. A proteomic study utilizing the PIMT-KO mouse revealed CKB as one of 22 major targets for PIMT in the brain [9].

There is considerable interest in how isoAsp formation leads to disruption of CNS function. Individual variation in PIMT activity has been linked to successful aging in humans and resistance to heat and metabolic stress in model organisms such as *D. melanogaster* and *C. elegans* [36–38]. Given the phenotype of the PIMT-KO mouse, extreme deficits in PIMT in humans would likely lead to neurodevelopmental disorders of the young, while moderate deficits might lead to an acceleration of age-related decline in CNS function. The purpose of the current study was to corroborate the identity of CKB as a physiological target of PIMT and determine if CKB catalytic function is altered by isoAsp formation. We report here that CKB activity is significantly decreased in parallel with its accumulation of isoAsp sites both *in vivo* and *in vitro*. Moreover, the isoAsp accumulation and activity loss can be substantially reversed *in vitro* with PIMT. The significance of these findings is enhanced by noting the marked similarity in phenotypes of the PIMT-KO and CKB-KO mice.
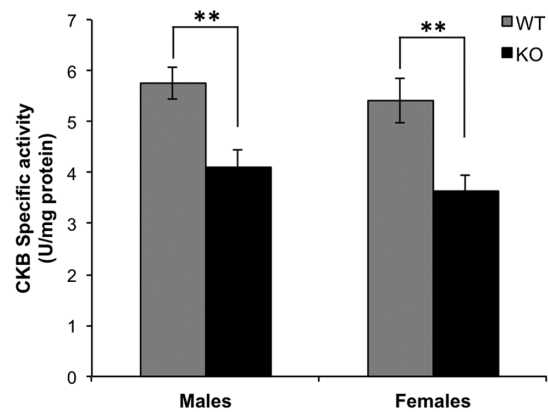

Figure 2. Determination of CKB specific activity in brain extracts from 4-week mice. CKB activity and protein concentrations were determined as described in Materials and Methods. Data are expressed as means ± S.D ($n = 5$ for each genotype). **$P < 0.01$. doi:10.1371/journal.pone.0100622.g002

Material and Methods

Reagents

S-Adenosyl-[methyl-3H]-L-methionine (3H-AdoMet) was purchased from PerkinElmer Life Sciences. A rabbit polyclonal antibody raised against full-length human recombinant CKB was purchased from Abnova (#PAB19125). A monoclonal rabbit antibody to β-actin (used as a loading control) was from Cell
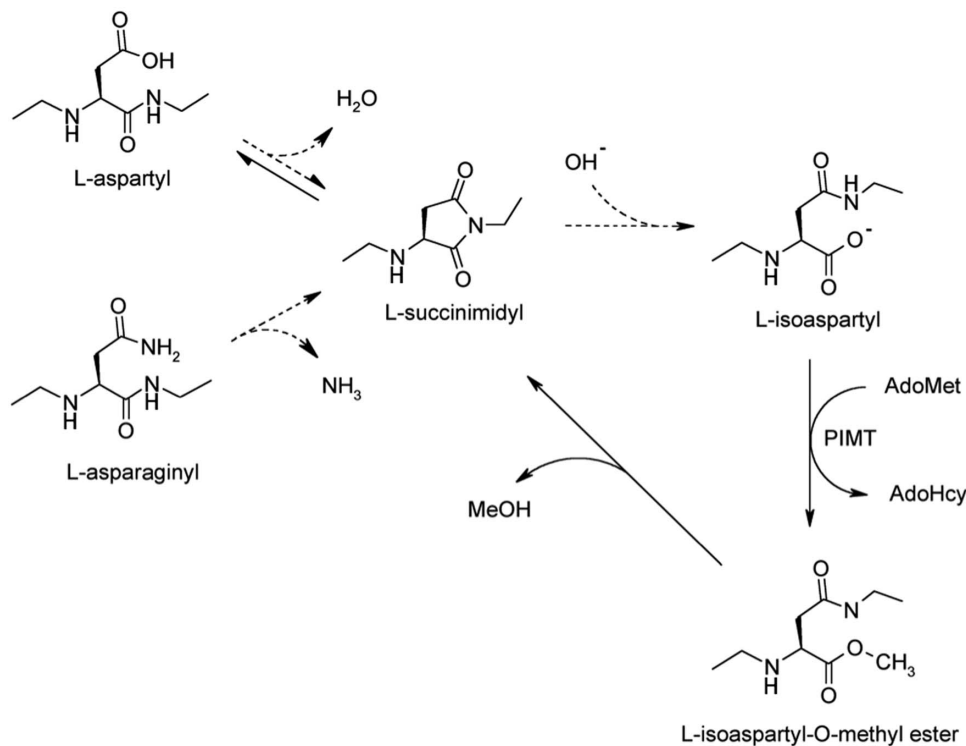
Figure 1. Mechanism of isoaspartate formation and PIMT-catalyzed repair. Under physiological conditions, deamidation of asparagine residues or dehydration of aspartic acid residues results in the formation of a metastable intermediate succinimide which spontaneously hydrolyzes to form a mixture of normal L-aspartyl and atypical L-isoaspartyl linkages. PIMT, using AdoMet as a methyl donor, selectively methylates the isoaspartyl α-carboxyl group to form a highly labile methyl ester. Spontaneous demethylation occurs within minutes to reform the original succinimide, with release of methanol as a by-product. This succinimide is now the starting point for further cycles of repair, resulting in near complete conversion of the isoaspartyl β-linkages to normal aspartyl α-linkages. doi:10.1371/journal.pone.0100622.g001

Signaling (#4970). Secondary antibody (horse radish peroxidase-linked donkey anti-rabbit IgG) and Pierce ECL Plus Western Blotting Substrate were purchased from GE/Amersham and Thermo/Pierce, respectively. Recombinant rat PIMT was expressed in *E. coli* and purified as described previously [9]. Recombinant human CKB was purchased from MyBioSource. Isoaspartyl delta-sleep inducing peptide (WAGGD^ASGE; where

^ designates an isopeptide bond) was purchased from Bachem.

Mice

PIMT$^{+/-}$ (HZ) founder mice were a gift of Dr. Mark Mamula (Yale University, New Haven, CT) and were originally generated by inserting a neo cassette into exon one of the pcmt1 gene [27]. PIMT$^{-/-}$ (KO) and PIMT$^{+/+}$ (WT) C57BL/6 mice were obtained by intercrossing PIMT-HZ mice. Genotyping was determined by tail DNA PCR analysis recognizing both the neo cassette and pcmt1 gene (Transnetyx, Inc., Cordova, TN). All animal housing and procedures were performed using a protocol approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Mice were anesthetized with a lethal dose of Euthasol prior to decapitation at 4 weeks of age.

Preparation of Mouse Brain Extracts

Mouse brains were weighed immediately after removal and suspended in 9 vol of ice-cold homogenization buffer (10% (w/v) sucrose, 5 mM K-Hepes, pH 7.6, 0.5 mM EDTA, 0.1 mM DTT (dithiothreitol), 50 mM NaF and 1 mM Na$_3$VO$_4$ as phosphatase inhibitors, and % (v/v) mammalian protease inhibitor mixture (Sigma). The suspension was homogenized on ice using a Potter-Elvejhem tissue homogenizer and then centrifuged at 800× g for

Figure 3. Expression of CKB in brain extracts is unaffected by PIMT genotype. (A). Representative Western blot of PIMT and β-actin expression in brains extracts from PIMT WT, HZ and KO mice. (B) Western blot quantitation after normalization of CKB bands to β-actin bands from the same sample. Data are expressed as means ± S.D (n = 5 for each genotype) relative to the WT male samples.
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30 min. The supernatant was centrifuged at 20,000× g for 1 h to pellet the mitochondrial CK fraction [39]. The final supernatant (hereafter "brain extract") was stored in aliquots at −80°C, and used as needed for determination of cytosolic CKB specific activity and assessment of CKB expression by Western blotting.

Protein Concentration and Enzyme Activity

Protein concentration was determined using the Pierce BCA microplate assay with bovine serum albumin as a standard. CKB activity in brain extracts was determined in the direction of ATP synthesis with a commercial kit (Pointe Scientific, Inc.) that uses the coupled enzyme assay shown in scheme 1.

Reaction 1 (CK catalyzed): creatine phosphate + Mg-ADP > ATP + creatine

Reaction 2 (HK catalyzed): ATP + glucose > ADP + G-6-P

Reaction 3 (G6PDH catalyzed): G-6-P + NADP$^+$ >6-GP + NADPH + H$^+$

CK, creatine kinase; HK, hexokinase; G6PDH, glucose 6-phosphate dehydrogenase; G-6-P, glucose-6-phosphate; 6-GP, 6-phosphoglucono-lactone. One unit of CKB activity is defined here as one μmol of NADPH generated per min at 37°C under initial rate conditions.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

Brain extracts were subjected to SDS-PAGE on NuPAGE 4–12% gradient gels with MES running buffer, and gels were stained using a Colloidal Blue Staining Kit (all from Novex, Life Technologies). Proteins were transferred to polyvinylidene difluoride membranes (0.45 μm pore) by semi-dry electroblotting. These were incubated for 2 h at room temperature with primary antibody to CKB (1:500,000) or β-actin (1:10,000), followed by 1 h in secondary antibody (1:15,000). The chemiluminescent signals were recorded with a Nikon D700 camera and quantitated using NIH ImageJ [40].

In Vitro CKB Aging and IsoAsp Quantitation m

Recombinant human CKB (0.4 mg/ml) was incubated in a closed microfuge tube for 16 days at 37°C in aging buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA, 2% (v/v) glycerol, 0.05% (w/v) NaN$_3$). Measurement of isoaspartate was determined by a methanol diffusion assay [41,42]. In brief, proteins (100 pmol in a final volume of 50 μl) were incubated in methylation reaction buffer (100 mM sodium phosphate, pH 6.8, 4 mM recombinant rat PIMT, 100 μM [^{3}H]AdoMet (500 dpm/pmol), 5 mM EDTA and 0.2 mg/ml BSA) for 30 min at 30°C. The reaction was terminated by addition of an equal volume of stop solution (400 mM Na-borate pH 10.4, 4% SDS, 2% methanol), then 50% of the sample was transferred to an accordion-pleated filter paper lodged in a Titeseal cap. Immediately the cap was fitted to a shell vial containing 2.5 ml of Liquiscint (National Diagnostics), and incubated for 1 h at 40°C prior to liquid scintillation counting. Isoaspartyl delta-sleep inducing peptide (50 pmol) was used as an internal standard for isoAsp quantitation. Optimization experiments have demonstrated that the conditions specified above result in quantitative methylation of the isoaspartyl peptide standard as judged both by ^{3}H-methanol recovery and by high-performance liquid chromatography [43].

Because isoAsp quantitation was carried out on intact CKB, isoAsp sites that are buried in the normally folded protein would not be detected. Buried sites can be measured if a protein is first rendered into peptide fragments by protease treatment [44,45]; but this was deemed unnecessary as this study is focused on the
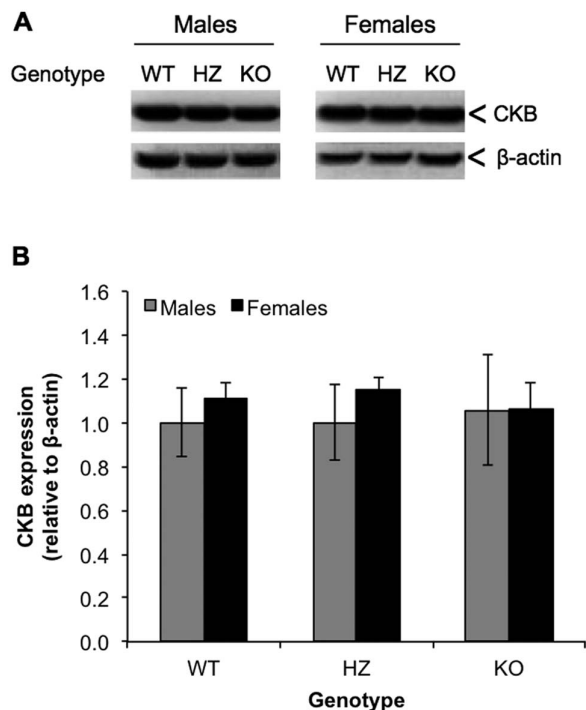
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CHK MPFSNSHNLLKMKYSVDDEYPDLSVHNNHMAKVLTLDLYKKLRDRQTSSGFTLDDVIQTG   60
MUS MPFSNSHNTQKLRFPAEDEFPDLSSHNNHMAKVLTPELYAELRAKCTPSGFTLDDAIQTG   60
HUM MPFSNSHNALKLRFPAEDEFPDLSAHNNHMAKVLTPELYAELRAKSTPSGFTLDDVIQTG   60
    ********  *:::  .:**:**** ********* :** :** : * *******.****

CHK VDNPGHPFIMTVGCVAGDEESYEVFKELFDPVIEDRHGGYKPTDEHKTDLNADNLQGGDD  120
MUS VDNPGHPYIMTVGAVAGDEESYDVFKDLFDPIIEERHGGYQPSDEHKTDLNPDNLQGGDD  120
HUM VDNPGHPYIMTVGCVAGDEESYEVFKDLFDPIIEDRHGGYKPSDEHKTDLNPDNLQGGDD  120
    ******:*****.*********:***:****:**:*****:*:******* *******

CHK LDPNYVLSSRVRTGRSIRGFCLPPHCSRGERRAIEKLSVEALGSLGGDLKGKYYALRNMT  180
MUS LDPNYVLSSRVRTGRSIRGFCLPPHCSRGERRAIEKLAVEALSSLDGDLSGRYYALKSMT  180
HUM LDPNYVLSSRVRTGRSIRGFCLPPHCSRGERRAIEKLAVEALSSLDGDLAGRYYALKSMT  180
    *********************************.****.** *** **** *:****:.**

CHK DAEQQQLIDDHFLFDKPVSPLLLASGMARDWPDARGIWHNDNKTFLVWINEEDHLRVISM  240
MUS EAEQQQLIDDHFLFDKPVSPLLLASGMARDWPDARGIWHNDNKTFLVWINEEDHLRVISM  240
HUM EAEQQQLIDDHFLFDKPVSPLLLASGMARDWPDARGIWHNDNKTFLVWVNEEDHLRVISM  240
    :************************************************:*:**:*****

CHK QKGGNMKEVFTRFCTGLTQIETLFKSKNYEFMWNPHLGYILTCPSNLGTGLRAGVHIKLP  300
MUS QKGGNMKEVFTRFCTGLTQIETLFKSKNYEFMWNPHLGYILTCPSNLGTGLRAGVHIKLP  300
HUM QKGGNMKEVFTRFCTGLTQIETLFKSKDYEFMWNPHLGYILTCPSNLGTGLRAGVHIKLP  300
    **************************:*******:*********************

CHK NLGKHEKFGEVLKRLRLQKRGTGGVDTAAVGGVFDVSNADRLGFSEVELVQMVVDGVKLL  360
MUS HLGKHEKFSEVLKRLRLQKRGTGGVDTAAVGGVFDVSNADRLGFSEVELVQMVVDGVKLL  360
HUM NLGKHEKFSEVLKRLRLQKRGTGGVDTAAVGGVFDVSNADRLGFSEVELVQMVVDGVKLL  360
    .*******.***************************************************

CHK IEMEKRLEKGQSIDDLMPAQK  381   P05122   KCRB_CHICK
MUS IEMEQRLEQGQAIDDLMPAQK  381   Q04447   KCRB_MOUSE
HUM IEMEQRLEQGQAIDDLMPAQK  381   P12277   KCRB_HUMAN
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Figure 4. Sequence alignment of chicken (CHK); mouse (MUS) and human (HUM) CKB. Six canonical hotspots for isoAsp formation are indicated with carets (^). Color-coding indicates regions of β-sheet (blue), α-helix (magenta), and turns (yellow) as deduced from crystal structure data according to the ExPASY (www.expasy.org) alignment application. Mouse is not color-coded because there is no crystal structure for mouse CKB. The dashed box encloses the acidic EED triplet essential for enzymatic activity.
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status of isoAsp sites that are accessible to PIMT-dependent repair. Moreover, previous studies with aged recombinant protein pharmaceuticals indicate that proteolytic digestion generally results in only a modest increase in measurable isoAsp sites [45]. This is consistent with the idea that isoAsp forms preferentially in flexible regions of a protein, and that such regions are typically found on the surface of a protein with good solvent exposure.

CKB specific activity was determined as described above for brain extracts.

In Vitro Repair of Isoaspartyl Sites

Repair reactions were carried out for 6 h at 37°C as described previously [23,46] in Pierce Slide-A-Lyzer microdialysis tubes (7-kDa cutoff) in a final volume of 50 μl. After final dialysis, CKB activity and isoAsp content were determined as described above. Methylation reactions used for repair are much longer in duration (6 h) than methylation reactions used for isoAsp quantitation (30 min), because the former requires multiple cycles of methylation/demethylation due to the inefficient yield of normal aspartyl peptide product when the succinimide hydrolyzes (Fig. 1).

Statistical Evaluation

Results are expressed as means ± standard deviations (S.D.) for the number (n) of samples. Statistical significance was calculated using the Student's t-test. Differences between means were considered significant if p-values were less than 0.05.

Results

CKB Activity is Reduced in PIMT-KO Mice

A previous study from our laboratory revealed that CKB appears to be a major substrate for PIMT in mouse brain [9]. To assess the effect of in vivo isoAsp accumulation on CKB function, the specific activity of CKB was determined in cytosolic extracts of PIMT-WT and -KO mouse brain (Fig. 2). The absence of PIMT reduced CKB activity by 28.9% in male mice, and by 32.9% in females. We tested both sexes because we recently found very significant differences in male vs. female mouse brains with regard to the phosphorylation of synapsin 1 (another major target for PIMT) [47], and because recent findings by others have demonstrated that sex differences in brain chemistry are pervasive and unpredictable [48]. The mean CKB activity in females was lower than in males (6.0% for WT and 11.3% for KO), but the significance was low (p>0.1).

We also compared CKB activity in mice that were WT vs. HZ with regard to the PIMT gene. PIMT activity in HZ brain extracts is 50–55% of that found in the WT, but HZ mice live a normal life span and exhibit no obvious phenotype. Using female mice only,
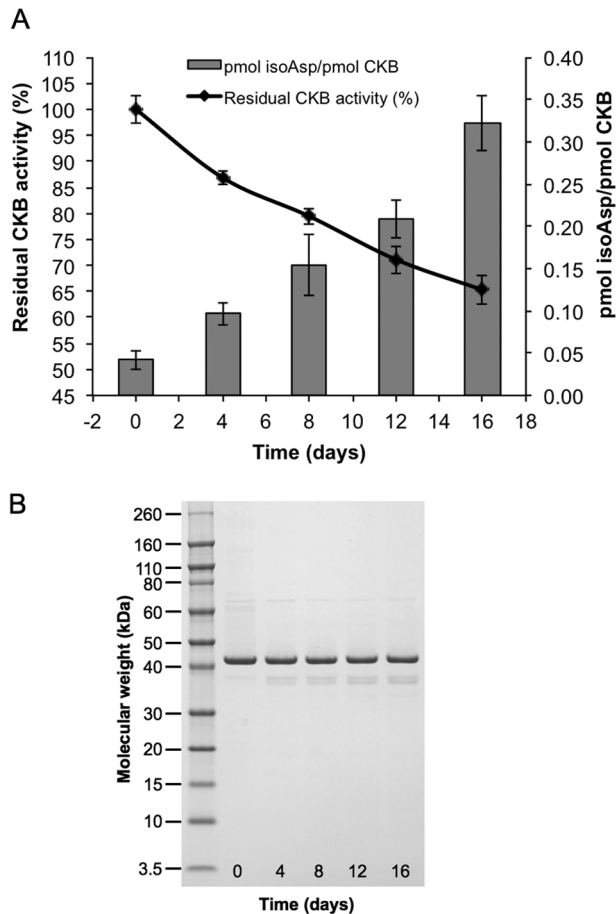
Figure 5. Effect of *in vitro* aging on enzymatic activity and isoaspartate content of recombinant human CKB. Recombinant human CKB (0.4 mg/mL) was aged *in vitro* at pH 7.5 and 37°C for 16 days. (A) At four-day intervals, samples were taken to assay CKB activity and isoAsp levels. Assays were performed in triplicate with standard deviations indicated by error bars. (B) SDS-PAGE of *in vitro* aged human recombinant CKB showing lack of significant degradation or aggregation.
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we found the average CKB activity in HZ mice was 8% lower than in WT, but again the results did not reach statistical significance.

Expression Levels of CKB in PIMT-WT, -HZ, and -KO Mice

To determine if the reduced CKB activity observed above in the PIMT-KO mice might be due to differential expression, we assessed levels of CKB protein in PIMT-WT, -HZ, and -KO male mice by Western blot using a polyclonal antibody to full-length human recombinant CKB. Because of the similar molecular weights of CKB (42.6) and β-actin (41.7 kDa; the loading control), the same samples were analyzed separately for β-actin in parallel Western blots. The results show that CKB is equally expressed in the brains of all three genotypes (Fig. 3). We conclude that the reduced CKB activity observed in the PIMT-KO mice is likely the result of CKB damage arising as a consequence of isoAsp formation.

Susceptibility of Recombinant Human CKB to In Vitro Aging

IsoAsp formation is an autocatalytic event (Fig. 1) that appears to go through the same succinimide mechanism both *in vitro* and *in*
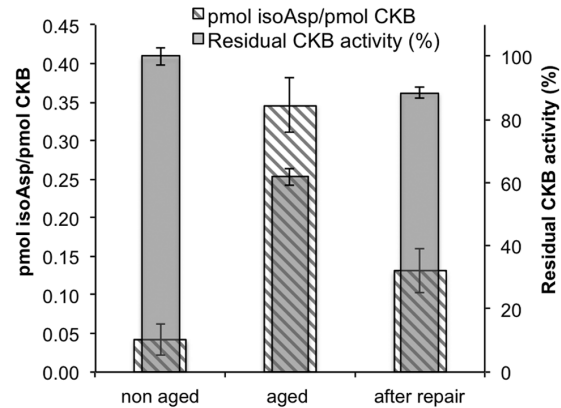

Figure 6. *In vitro* repair of the isoaspartyl sites in aged recombinant CKB. Aged recombinant human CKB was subjected to *in vitro* repair as described under "Materials and Methods" in the presence of PIMT and unlabeled AdoMet. CKB activity (right axis) before aging, after aging and after repair was determined and isoAsp content (left axis) was measured by a methanol diffusion assay. Each assay was performed in triplicate with error bars representing standard deviation.
doi:10.1371/journal.pone.0100622.g006

vivo [49]. Our observation of reduced CKB activity in the PIMT-KO mouse thus predicts that purified CKB should, during *in vitro* aging at physiological pH and temperature, undergo measurable isoAsp accumulation with concomitant loss of enzymatic activity. We therefore subjected human recombinant CKB (which is commercially available and shares high sequence homology to mouse CKB; Fig. 4) to *in vitro* aging for 16 days at pH 7.5 and 37°C and then assayed for both isoAsp content and enzyme activity (Fig. 5A). CKB accumulated high levels of isoAsp (0.33 mol isoAsp/mol CKB), which was accompanied by a 34% loss of initial enzyme activity. To look for possible aggregation or proteolytic degradation that would complicate interpretation of these results, the aged samples were subjected to SDS-PAGE and subsequent staining with Coomassie Colloidal Blue (Fig. 5B). Staining revealed the expected dominant band at 43 kDa (CKB), a faint band at ~70 kDa that did not change over time, and a pair of faint bands at ~38 kDa whose intensity increased slightly over time. The near constant intensity of the CKB band at 43 kDa indicates that the enzyme remains substantially intact over the aging period, supporting the conclusion that loss of activity is due to mainly isoAsp formation, and not degradation or aggregation of intact CKB.

PIMT Repairs IsoAsp Sites in Aged Recombinant CKB

Several previous studies have shown that purified PIMT catalyzes the conversion of isoaspartyl peptide bonds to normal α-carboxyl-linked peptide bonds in peptides and proteins. We tested the potential of PIMT to repair isoAsp sites and restore activity in recombinant CKB that had been subjected to *in vitro* aging for 16 days as described above. To do this we used a protocol in which the age-damaged protein is incubated for 6 h with PIMT and unlabeled AdoMet while being dialyzed against a relatively large volume of AdoMet [23,46]. This dialysis procedure facilitates repair by constantly replenishing the AdoMet methyl donor, while preventing the accumulation of its by-product, S-adenosyl-L-homocysteine (a potent inhibitor of PIMT). After further dialysis to remove unlabeled AdoMet, portions of the analyte (in this case CKB) are methylated with ^{3}H-AdoMet to assess its isoAsp content, and also assayed for enzymatic activity. As shown in Fig. 6, we found that the repair reaction restored approximately 69% of the CKB enzyme activity that was lost

Table 1. Comparison of phenotypes of PIMT-KO and CKB-KO mice, relative to wild type mice.

Characteristic	PIMT-KO	CKB-KO[a]
Habituation: thigmotaxis[b]	"hyperactivity in the open-field test" and "a strong thigmotaxic movement pattern" (Vitali and Clarke, 2004)	"diminished open-field habituation" and "none of the (KO) mice created a home-base"
Learning: Morris water maze	"impaired spatial memory" (Ikegaya et al., 2001)	"slower to learn, but acquired the spatial task"
Epileptic seizures	Highly susceptible. Spontaneous fatal seizures at 1–3 mo. (Kim et al., 1997)	In response to pentylentetrazole, the KO mice "showed significantly more myoclonic jerks preceding the first seizure…and between the first and second seizures."
Coordination: accelerating rotarod	"perform significantly better than their heterozygous and wild type litter-mates' (Vitali and Clarke, 2004)	"did not differ from wild type mice in their motor coordination and balance on the rotarod"
Hippocampal histology	"increased cell proliferation and granule cell number in the dendate gyrus" (Farrar et al., 2005)	"intra- and infra-pyramidyl mossy fiber field is larger in (KO) mice"

[a]All of these entries are from Jost et al., 2002.
[b]A taxis in which contact, especially with a solid body, is the directive factor.
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during aging, while eliminating approximately 57% of the isoAsp sites that had accumulated during aging.

Discussion

Creatine kinase B is highly susceptible to isoAsp formation, as demonstrated by proteomic analysis of major methyl-accepting substrates for PIMT in PIMT-KO mouse brain extracts. The phenotype of this KO mouse includes learning deficits, lack of normal habituation, and fatal epileptic seizures at 4–8 weeks after birth. Our goal in the present study was to determine if isoAsp damage to CKB might contribute to the neuropathology resulting from PIMT deficiency. IsoAsp accumulation *per se* does not always lead to loss of biological function, as exemplified by the case recombinant human growth hormone [50], so it is of interest to determine the extent to which major *in vivo* targets of the PIMT repair enzyme actually undergo reduced biological function due to isoAsp formation. The data we present here strongly suggest that isoAsp formation in mouse CKB dramatically reduces its enzymatic function. The association between isoAsp levels and reduced activity was demonstrated with isoAsp formation occurring both *in vivo* and *in vitro*. Moreover, we were able to demonstrate that incubation of isoaspartyl-damaged CKB regains significant function when incubated with PIMT and its methyl donor AdoMet. Overall, these results suggest that CKB functional damage contributes to the neuropathology characteristic of the PIMT-KO mouse.

PIMT-KO mice and CKB-KO mice have overlapping phenotypes

Similarities between PIMT-KO and CKB-KO mice further supports the idea that decreased CKB function contributes to the PIMT-KO phenotype. Table 1 compares the phenotypic analysis of CKB-KO mice carried out by Jost et al. [5] with corresponding studies carried out by various researchers on the PIMT-KO mouse. In short, these mice are remarkably similar with regard to (a) thigmotaxis (atypical persistent walking around the perimeter of a novel enclosure, signifying reduced habituation), (b) decreased learning in the Morris water maze test, (c) enhanced susceptibility to epileptic seizures, (d) abnormal histology in the hippocampus,

and (e) normal (CKB-KO) or supra-normal (PIMT-KO) performance in the accelerating rotarod coordination test.

Overall, the CKB-KO phenotype is milder than the PIMT-KO phenotype. Susceptibility to seizures is much lower in the CKB-KOs, and they live a normal life span. This difference in phenotypic severity is expected as numerous proteins (probably 50 or more) are significantly affected in the PIMT-KO. Another major *in vivo* target of PIMT, synapsin I, shows evidence of functional deficits as evidenced by its *in vivo* state of hyperphosphorylation relative to the PIMT-WT mouse [47]. Moreover, the synapsin-KO mouse also has a phenotype (learning deficits and increased susceptibility to seizures) that overlaps with the PIMT-KO mouse.

CKB contains several predicted hotspots for isoAsp formation

The sequences of mouse and human CKB (Fig. 4) reveal that each has six potential "hotspots" for isoAsp formation; Asx residues followed by Gly, Ser, or His. At physiological pH and temperature, isoAsp forms most rapidly at these sequences in short synthetic peptides, and is often found in age-damaged proteins, especially when these sequences occur in flexible domains. Of these six hotspots in CKB, four are located at the border of an α-helix. Asp-190 is at the trailing edge of an α-helix (181–190) located on the surface of the molecule, in one of three flexible regions that undergo large conformational changes during catalysis [51]. Asp-233 forms a hydrogen bond with the backbone amide of Thr-282, fixing the shape and stereochemistry of the active site, and is close to Arg-236, another residue essential for catalysis.

Phosphorylation is a common regulatory modification in proteins. A comprehensive survey of the human phosphoproteome has revealed that creatine kinase B has four phosphorylation sites; Ser-4, Ser-6, Thr-35, and Ser-163 [52]. The exact function of these modification sites has yet to be determined, but it is interesting to note that one of the putative hotspots for isoAsp formation in CKB (Asn-5) is sandwiched between two of these phosphorylation sites. How phosphorylation and isoAsp formation might affect each other at this locus in CKB is an attractive question for future research.

IsoAsp formation may promote in vivo oxidation of, and autoimmunity to, CKB

CKB is prone to oxidative damage *in vivo* and can also serve as an autoantigen [53,54]. A number of recent studies suggest oxidation and isoaspartate formation interact synergistically. Oxidation of purified hemoglobin with acetylphenylhydrazine produces a rapid increase in its isoAsp content [55], and exposure of erythrocytes to hydrogen peroxide has been found to induce isoAsp formation in membrane proteins [56]. UVA radiation has been found to trigger isoAsp formation in melanoma cells, while natural antioxidants such as hydroxytyrosol (found in olive oil) protect melanoma cells against both oxidative damage and isoAsp accumulation [57]. Oxidation of ceruloplasmin promotes conversion of asparagine to isoAsp in its two Asn-Gly-Arg (NGR) sites, thereby creating a motif (isoDGR) that can recognize the RGD-binding site of integrins [58,59]. Cellular levels of PIMT can greatly affect the susceptibility of cells to apoptosis induced by oxidative stress, as observed in cultured human endothelial cells and the nematode *C. elegans* [38,60]. This synergism between two common forms of protein damage suggests a potential role for isoAsp formation in oxidation of CKB *in vivo*. We note here that the histidines adjacent to Asn-28 and Asp-190 are susceptible to oxidative modification by 4-hydroxy-2-nonenal, a byproduct of peroxidation that is increased in Alzheimer's disease [61]. The structural basis for synergism between oxidation and isoAsp formation probably results from the fact that either type of damage can destabilize protein structure, which in turn can render a protein susceptible to further damage by the other mechanism.

A survey of retinal autoantigens associated with endogenous uveitis identified CKB, β-tubulin, β-actin, aspartate aminotransferase, and the voltage-dependent anion-selective channel protein as major contributors [62]. Interestingly, all four of these proteins are among the 22 polypeptides we detected as major endogenous substrates for PIMT in the KO mouse brain. Mamula and colleagues have shown that the presence of isoAsp can greatly increase a protein's ability to break tolerance in the innate immune system [14,63]. Thus, it appears that isoAsp formation can have multiple deleterious effects on CKB: loss of catalytic activity, increased susceptibility to oxidation, and conversion to an autoantigen.

Author Contributions

Conceived and designed the experiments: AD DWA. Performed the experiments: AD ZQ. Analyzed the data: AD ZQ DWA. Contributed reagents/materials/analysis tools: DWA. Contributed to the writing of the manuscript: AD ZQ DWA.

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